University of South Bohemia in České Budějovice, Faculty of Science

Institute of Chemistry and Biochemistry

Iron-Sulfur Cluster Assembly in *Trypanosoma brucei*

Bachelor thesis

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České Budějovice, 2013

Haindrich A. C., 2013. Iron-Sulfur Cluster Assembly in *Trypanosoma brucei*. Bc. thesis in English, 52 p, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

Annotation

The aim of this thesis was to investigate genes of the Cytosolic Iron sulfur cluster Assembly (CIA) pathway in *T. brucei* procyclic and blood-stream form for their possible functional redundancy.

Annotation

Das Ziel dieser Arbeit war die Untersuchung von Genen des cytosolischen Eisen Schwefel Cluster Syntheseweges auf mögliche funktionelle Redundnanz, in der prozyklischen und metazyklischen trypomastigoten Form von *T. brucei*

Affirmation

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act. No. 111/1998. I also agree to the comparison of the text of my thesis with the Thesis.cz thesis database operated by the National Registry of University and a plagiarism detection system.

České Budějovice, 16. 05. 2013

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Alexander Haindrich

Acknowledgements

I would like to express my thanks to Prof. Julius Lukeš for giving me the opportunity to work in his laboratory, the great working climate I experienced in his lab and for giving me new faith in molecular biology after my PCR failed for the 10th time.
I also would like to thanks Eva Horáková as my group leader for the smooth guidance into reading of scientific papers, and for the many times I had to borrow her time when me and Som had an, for us, unsolvable problem or to get a third opinion on a decision to make.
Which bring me to my supervisor Somsuvro Basu, I express my thanks to him for his great guidance into molecular biology working routine, for his flawless performance as tutor, his trust in my shaky hands, the flexible working I could conduct under his watchful eye, his scientific support in all my questions and I would also thank him for being my friend.
I also need to express my gratitude to the whole remaining team of the Lukeš lab, because I think during the course of the work on my thesis I didn't leave out any single one from asking at least for some advice, help or something I needed.

Abbreviations

bp	base pairs
c-aconitase	cytosolic aconitase
CIA	cytosolic iron-sulfur cluster assembly
DK	double-knockdown
E. coli	*Escherichia coli*
EtBr	ethidium bromide
FP	forward primer
[Fe-S]	iron-sulfur cluster
G418	geneticin
Hyg	hygromycin
ISC	iron-sulfur cluster system
NIF	nitrogen fixation system
PCR	polymerase chain reaction
Phleo	phleomycin
RNAi	RNA interference
SK	single knockdown
SUF	sulfur utilization factor system
Ta	annealing temperature
T. brucei	*Trypanosoma brucei*

Introductory note

The genes studied in this thesis are named after their yeast orthologues. When the yeast gene name is used and no additional information is given it is revered to the *Trypanosoma* orthologue or it is specified which gene of which organism is addressed. Following genes have been studied:

Table 1: Summary of gene names of CIA components in *S. cerevisiae* and Human and their predicted orthologues in T. brucei with their gene ID as they are found on tritrypdb.org

Protein name used in thesis	*T. brucei* homolog Gene ID.	*S. cerevisiae* homolog	Human homolog
Cia1	Tb927.8.3860	Cia1	CIAO1
Cia2A*	Tb927.8.720	YHR122W (old Cia2)	Fam96B (Fam96A)
Cia2B*	Tb09.211.1780		
Dre2	Tb927.8.1750	Dre2	CIAPIN1 (Anamorsin)
Met18	Tb927.8.3920	Met18	MMS19
Nar1	Tb927.10.10650	Nar1	IOP1
Nbp35	Tb927.10.1690	Nbp35	NUBP1
Tah18	Tb927.4.1950	Tah18	CPR

* Humans have two homologes of the yeast Cia2 protein, *T. brucei* also has two orthologues, but both of them are more similar to Fam96B than to Fam96A. Trypanosoma Cia2 was named after the yeast Cia2 homolog, and as there are two orthologues they were named Cia2A and Cia2B.

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1 Introduction

1.1 Trypanosomatids

1.1.1 General

Trypanosomatids are protozoa of the class kinetoplastida. They are uniflagellated and therefore easily distinguishable from the rest of the kinetoplastids. Trypanosomatids include the genera *Trypanosoma*, *Leishmania* and *Phytomonas*. All of them are strictly parasitic (Moreira, López-García, & Vickerman, 2004), having hosts spread over all classes of vertebrates, several invertebrates and some plants (Overath, Stierhof, & Wiese, 1997). *Trypanosoma* and *Leishmania* are the causative agents off many important diseases such as human sleeping sickness and leishmaniasis. Many t*rypanosomatids* have complex life cycles including several hosts usually including an insect vector and a vertebrate host (Bringaud, Rivière, & Coustou, 2006). *Phytomonas* contains mainly plant parasites (Canepa, Carrillo, Miranda, Sayé, & Pereira, 2011).

1.1.2 *Trypanosoma brucei*

Trypanosoma brucei is the main parasite causing African trypanosomiasis. There are three different subspecies of *T. brucei* which cause different types of trypanosomiasis. *T. brucei rhodesiense* causes fast onset acute trypanosomiasis in humans in east and south Africa, whereby game animals and livestock are thought to be the primarily mammalian host. The second subspecies is *T. brucei gambiense* which affects mainly humans in central and west Africa. The disease induced by *T. b. gambiense* has a slower onset and leads to chronic trypanosomiasis or also called sleeping sickness. The third subspecies is *T. brucei brucei* which causes acute nagana or animal African trypanosomiasis in livestock. All three parasites have the tsetse fly (*Glossina* species) as their insect vector housing the parasites in their midgut or the salivary glands in their procyclic form, and are transmitted to their respective mammalian host during blood feeding (Barrett et al., 2003). There are another two subspecies of *T. brucei* worth noting, which are actually evolved from *T. brucei brucei*, namely *T. equiperdum* which causes dourine, and *T. evansi*, which causes surra, in horses, camels and water buffaloes. *T. equiperdum* has partly and *T. evansi* has totally lost their kinetoplastid DNA, and both of them are trapped in their blood stream form and can only be transmitted over the blood stream by blood sucking insects or by coitus (Lai, Hashimi, Lun, Ayala & Lukeš, 2008). *T. b. brucei* is often used as model organism for the study of the

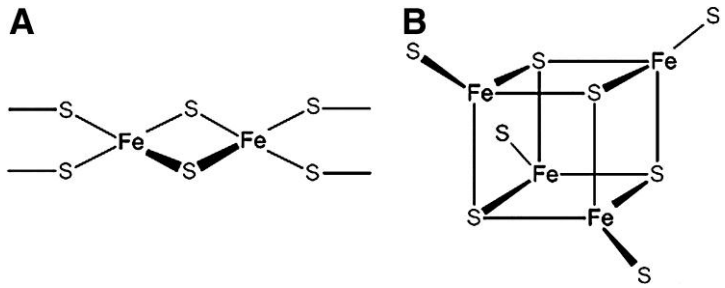
human pathogens *T. b. rhodesiense* and *T. b. gambiense*, being similar to this species and non-infectious to humans because of their susceptibility to lysis by apolipoprotein L-I in human serum (Vanhamme et al., 2003). *T. brucei* is easily cultivated in the laboratory in both of its life stages, furthermore its complete genome has been sequenced in 2005 (Berriman et al., 2005), and various genetic tools like RNAi or gene knock-out generation are available, which makes *T. brucei* a suitable model organism for the investigation of the function of a variety of proteins of interest (Montagnes, Roberts, Lukeš & Lowe, 2012).

1.2 Iron-sulfur cluster and Iron Sulfur Cluster Proteins

1.2.1 General

Iron-sulfur clusters [Fe-S] are evolutionary ancient small inorganic cofactors present in all domains of life and are involved in various biochemical reactions and functions. Despite their fundamental role in biology [Fe-S] was first discovered only in 1960.
[Fe-S] are present in nature with different stoichiometric ratios of iron and sulfur, the most common ones are the rhombic [2Fe-2S] and the cubic [4Fe-4S] which are often incorporated into [Fe-S] proteins. In some [Fe-S] proteins an iron of a [4Fe-4S] may be lost to generate a [3Fe-4Fe].

Figure 1: The structure of the two most commonly found [Fe-S]: (A) rhombic and (B) cubic clusters [5]



The iron in the clusters can have an oxidation state of +2 or +3 while the sulfur always has the oxidation state -2. This ability of the iron atom to undergo changes in its oxidation state makes [Fe-S] very suitable as mediator in biological redox reactions and for electron transport. Indeed the reduction potential of [Fe-S] ranges from +300mV to -500mV giving them a broad operational range involving photosynthesis, respiratory chain, nitrogen fixation, redox catalysis, DNA replication and repair, regulation of gene expression, tRNA

modification and with ongoing research are found to be involved in even more compartments of life.

The first [Fe-S] emerged when there was still an anaerobic, oxygen poor environment on Earth and little is known about the first appearance of [Fe-S] proteins, but it is thought that they had essential roles in DNA metabolism and as electron transporter since the beginning of life. [Fe-S] are however very vulnerable to oxidation by oxygen, and with increase of atmospheric oxygen produced by cyanobacteria, [Fe-S] wouldn't be stable any more. This was an evolutionary reason for the incorporation of [Fe-S] into proteins to give them a protective shield against the new emerged oxygen environment.

[Fe-S] proteins are built up from an apo-protein which is the [Fe-S] protein without the cluster and an [Fe-S] cluster is required to form the so called holo-form of the [Fe-S] protein. While the [Fe-S] itself can be assembled *in vitro* with low effort and even be transferred onto apo-proteins, but nature requires more sophisticated ways to assemble the [Fe-S] and transfer them to [Fe-S] proteins. Over the past years of [Fe-S] research several such biogenesis pathways were discovered. In bacteria three [Fe-S] biogenesis systems are known: the nitrogen fixation system (NIF), the iron-sulfur cluster system (ISC), and the sulfur utilization factor system (SUF). Eukaryotes possess an ISC-like system which takes place mainly in mitochondria and additionally the cytosolic iron-sulfur protein assembly (CIA) which takes place in the cytosol. Photosynthetic eukaryotes exhibit a third system which takes place in the chloroplasts similar to the bacterial SUF system (Lill, Ulrich & Mühlenhoff, 2008; Xu & Møller, 2011).

1.2.2 Cytosolic iron-sulfur cluster protein assembly (CIA)

The presence of a cytosolic [Fe-S] protein assembly pathway was first discovered in *S. cerevisiae*. The CIA is not capable of assembling [Fe-S] solely one its own but it depends on the mitochondrial cysteine desulfurase Nfs1p. Nfs1p is the yeast orthologue of the bacterial cysteine desulfurase NifS which is part of bacterial NIF system where it is required for the production of elemental sulfur. The sulfur produced by Nfs1p gets exported from the mitochondria by the ATP-binding (ABC) transporter Atm1p in a still unknown form termed compound X. Nfs1p showed to be essential for cytosolic [Fe-S] assembly as well as for mitochondrial [Fe-S] biogenesis, while depleting yeast cells of Atm1p only affects cytosolic [Fe-S] protein assembly. (Kispal, Csere, Prohl, & Lill, 1999). Initially it was thought that all

[Fe-S] are solely assembled in mitochondria, but these findings suggested that there must be another [Fe-S] assembly pathway located in the cytosol.

The discovery of the cytosolic iron sulfur cluster protein assembly pathway was materialized during the investigaton of the assembly of cytosolic aconitase from iron regulatory protein 1 (IRP1). C-aconitase was known to possess a [4Fe-4S], and it can be converted from its apo-form (IRP1) to its holo-form (c-aconitase) *vice-versa* by assembly or disassembly of the [Fe-S]. These investigations lead to the discovery of the cytosolic Fe-S cluster deficient (CFD) gene. The gene codes for a highly conserved, putative P-loop ATPase (Cfd1p). Cfd1p has been shown to be localized in the cytosol and mutation of Cfd1 leads to significantly reduced aconitase activity, making Cfd1p the first cytoplasmic [Fe-S] assembly factor described in eukaryotes (Roy, Solodovnikova, Nicholson, Antholine & Walden, 2003). The link of aconitase to the CIA pathway also led to the possibility of testing aconitase activity levels in cell lysates as a marker to quantify [Fe-S] protein activities connected to the CIA pathway.

Cfd1p possesses a homologue NBP35p which is also localized in the cytosol as well as in the nucleus and is also involved in CIA. NBP35p additionally contains four conserved cysteine residues in its N-terminus those coordinate a [Fe-S]. (Hausmann et al, 2005). In yeast NBP35p forms together with Cfd1p a heterotetrameric complex that can associate [4Fe-4S] on its C-termini. This [Fe-S] cluster is then transferred by Nar1 further to be incorporated in apoproteins (Urzica, Pierik, Mühlenhoff & Lill, 2009). Dre2 provides to the Cfd1-Nbp35 complex a still unknown form of sulfur, through the ABC transporter protein Atm1 from the ISC machinery. It is evidnt from this information that the CIA machinery is dependent on the ISC machinery. The sulfur received by Dre2 from the ISC gets reduced by Tah18 (a diflavin reductase having both FAD & FMN domains) which transfers electron to Dre2, on the downstream the electrons it accepts from the reduction of NADPH. (Netz et al, 2010). The [Fe-S] is finally assembled on the Cfs1-Nbp35 complex, but also the source of iron used for the assembly of the cluster is still dubious, however the source is cytosol and not the mitochondria.

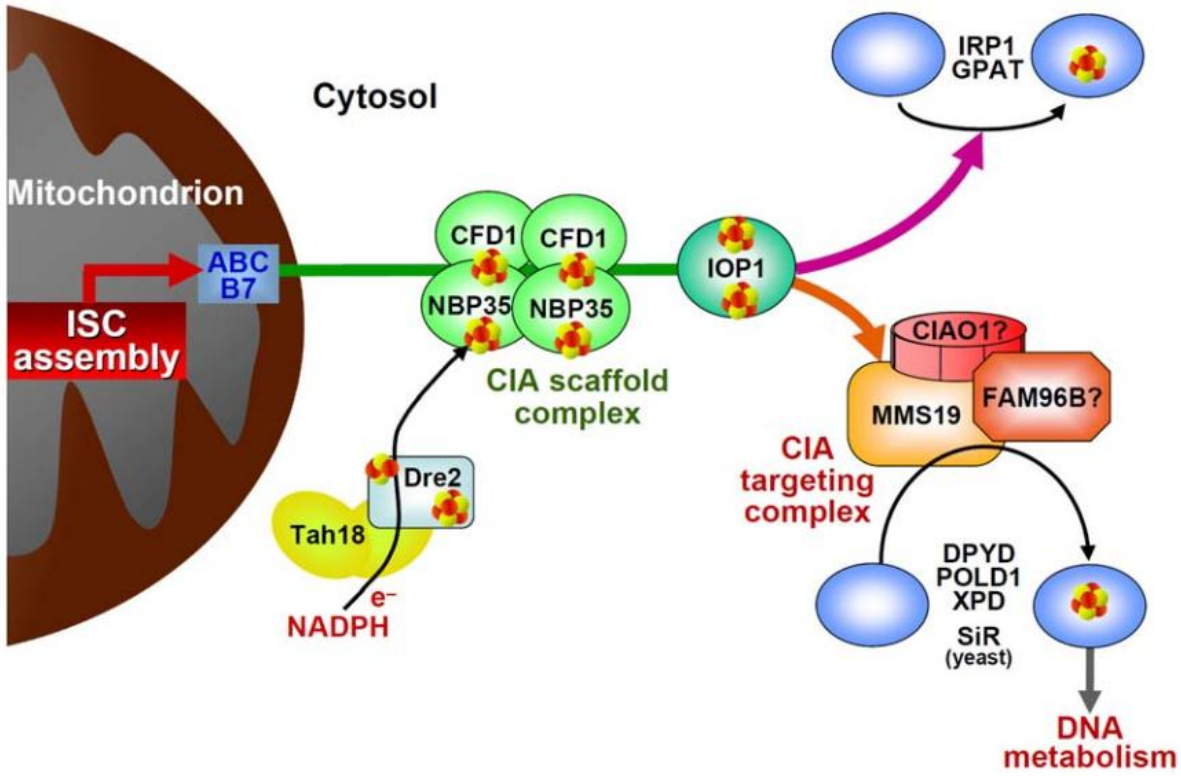


Figure 2: Current working model of cytosolic iron sulfur cluster assembly in human (Stehling et al. 2012)

The cluster gets completely synthesized on the Cfd1-Nbp35 complex. Because of the sensitivity of the [Fe-S] to degradation by oxygen further steps are needed to transfer the finished clusters, safely to the apo-protein. The model (Figure 2) suggests that after the cluster gets bound to Nar1 the pathway splits into two possible directions where Nar1 is the common transporter of the clusters. One way goes directly to assemble the holo-form of iron regulatory protein-1 (IRP1) which is also an isoform of cytosolic aconitase, and to glutamine phosphoribosylpyrophophate amidotransferase (GPAT). In the second path Nar1 transports the cluster to an CIA targeting complex consisting of Met18 (MMS19), Cia1 (CIAO1), and Cia2 (homologue of FAM96B). A pull down assay using Met18 as bait showed that this complex binds to a variety of iron-sulfur proteins involved in DNA metabolism and repair, like the DNA polymerase subunit POLD1, the helicase XPD or the [Fe-S] protein dihydropyrimidine dehydrogenase DPYD (Stehling et al. 2012).

1.2.3 Diseases related to [Fe-S] assembly and [Fe-S] proteins

[Fe-S] are essential for a wide range of proteins from energy metabolism to DNA repair. A genetic defect in the assembly of [Fe-S] therefore can have severe effects on cell functionality. There are at least five distinctive human diseases that are caused by malfunctioning proteins involved in the [Fe-S] assembly. These diseases include Friedreich´s

ataxia (FRDA) which is caused by an decreased expression of frataxin, GLRX5-deficient sideroblastic anemia caused by a low expression of GLRX5, ISCU myopathy causes by miss splicing of ISCU, Mitochondrial ecephalomyopathy caused by a mutation in NUBPL that results impaired respiratory complex I, and multiple mitochondrial dysfunctions syndrome caused by mis-expression of either NFU1 or BOLA3. For most of these diseases, except for Friedreich's ataxia which is present in 1/50000 births, only few cases are reported. One reasons behind this is, they are partly so severe that death already results a few weeks or months after birth and they remain therefore often undetected. The list of diseases linked to [Fe-S] is however growing (Rouault, 2012), giving further reasons for deeper investigation and understanding of this topic.

1.3 Used Techniques

1.3.1 RNA interference (RNAi)

RNA interference (RNAi) is a powerful tool that is nowadays widely integrated in normal cell biology. It was first described in *Caenorhabditis elegans* by Fire & Mello in 1998 who also were awarded the Nobel Prize for their discovery in 2006. It was already observed in plants earlier that expression of antisense RNA causes a transcriptional inhibition of the expression of the target gene. Fire & Mello however found out that neither sense nor anti-sense RNA, but double stranded RNA (dsRNA) causes the inhibition, and the expression of antisense RNA in plants also led to the formation of double stranded RNA with the original expressed sense RNA (Fire et al., 1998).

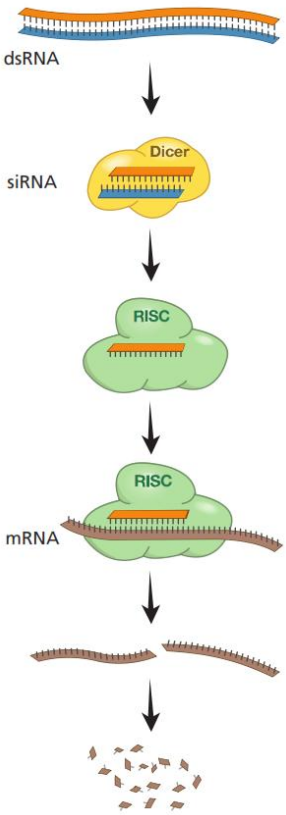


Figure 3: Schematic cartoon of the singl steps of RNAi (Advanced informations on The Nobel Prize in Physiology or Medicine 2006)

RNAi is a natural defense mechanism against RNA viruses found in a wide range of organisms. RNAi is initiated by the presence of double stranded RNA in the cell, which can either be introduced from the outside of the cell eg. by a virus, or overexpressing the dsRNA of a gene of interest in most reverse-genetic experimental set ups. The dsRNA becomes cleaved into shorter dsRNA fragments with a length of 21 to 23 nucelotides, by a ribonuclease III-like nucelase called dicer. These small dsRNA fragments are then also called small interfering RNA

(siRNA). The antisense RNA of this siRNA then gets loaded into a large complex called RISC (RNA-induced silencing complex). The RISC uses the anti-sense RNA to bind to the corresponding target mRNA. Once bound to the mRNA an endonucelase contained in the RISC cleaves the mRNA which subsequently becomes target for degradation and therefore lead to a silencing of the expression of the corresponding gene (Advanced informations on: The Nobel Prize in Physiology or Medicine 2006). This molecular biological tool allows the quick and specific knock-down of single genes on the level of their mRNA expression. The possibility of high throughput RNAi also allowed the loss-of function screening of whole genomes within months.

1.3.1.1 RNAi in *Trypanosoma brucei*

Short after the discovery of RNAi in *C. elegans* it was also found that *T. brucei* contains all necessary gene for a successfully working RNAi process (Ngô, Tschudi, Gull & Ullu, 1998). There are many different vectors available to produce dsRNA in *T. brucei* to trigger RNAi, but all of them can be separated by their working mechanism into two different groups. One group uses head-to-head T7 promoters that will overexpress sense and anti-sense RNA which then form the dsRNA (LaCount, Bruse, Hill & Donelson, 2000), the other uses a T7 promoter to express a stem loop RNA which contains a stuffer region between two inverted repeats of the target gene (Shi et al, 2000). The T7 RNA polymerase is usually linked to a Tet repressor which can be activated by tetracycline induction (Wirtz, Leal, Ochatt & Cross, 1999). This system of tetracycline induced RNAi in *T. brucei* makes it the method of choice for quick loss-of-function screens of genes with unknown function and allows to make first conclusion over the nature and function of the targeted gene. RNAi however doesn't work with all gene regions, and the results are therefore mostly viewed as strong evidences if positive, but not necessarily as proof for essentiality, and it is usually only used as basis for the design of further experiments.

2 Aims of the thesis

The aim of my thesis was to develop RNAi knockdown cell lines of *T. brucei* in its blood stream and its procyclic form, knocking down two selected genes of the cytosolic iron sulfur cluster assembly (CIA) pathway in tandems, to check for the possible functional redundancy between the RNAi knocked down partners. The double knockdowns were constructed from already existing single knockdown plasmids, and knockdown partners were selected based on the proposed model of the CIA pathway in *S. cerivisiae*. For all generated RNAi cell lines, growth effects upon tetracycline induced RNAi had to be measured.

3 Materials and Methods

3.1 Materials

3.1.1 Used Organisms

For the amplification of the plasmids which were used for the RNAi *Escherichia coli* XL1-Blue was used.

For the RNAi knockdowns in *Trypanosoma brucei* the strain 29-13 was used in its procyclic and in its blood stream form for the respective experiments investigating these stages of the parasite life cycle. The *T. brucei* strain 29-13 contains an integrated gene which codes for a T7 polymerase linked to a tetracycline repressor, and two antibiotic resistance gens coding for hygromycin and geneticin.

3.1.2 Primers used for Double Knock-down construction

The primers used for the construction of the RNAi double knockdown constructs were designed using the TrypanoFAN: RNAit web-tool (http://trypanofan.path.cam.ac.uk/software/RNAit.html), by my co-supervisor Somsuvro Basu, MSc.

Table 2: *Knockdown Primers*, SK…Single Knockdown, DK…Double Knockdown, FP… Forward Primer, RP…Reverse Primer, …Restriction Site, … Part of gene, Ta…Annealing Temperature used in PCR program, Product Size…Estimated size of PCR product when using forward and reverse primer

Name	Restriction Site	Sequence	Ta	Product size
Cia1 SK FP	BamHI	CGTGGATCCTATTTCTCGTGGATGGAGCA	50°C	324 bp
Cia1 SK RP	XhoI	CGTCTCGAGCCGGCTATGCTCACCTTCTA	50°C	
Cia2B SK FP	XhoI	CGTCTCGAGCGTTTAACGGCAGAGGATGT	58°C	418 bp

Cia2B SK RP	BamHI	CGTGGATCCCAACTTCCTGTAGAAGCGCC	58°C	
Dre2 SK FP	XhoI	CGTCTCGAGCACACAGGCCTTCAGTCTCA	58°C	312 bp
Dre2 SK RP	BamHI	TCAGGATCCTCCAACTTCACTTTCCCGTC	58°C	
Nar1 SK FP	Bam HI	CGTGGATCCATGTCGGCCAACAATTTCTC	58°C	464 bp
Nar1 SK RP	XhoI	CGTCTCGAGATCTCACTCGGCGACAGTA	58°C	
Cia1 DK FP	BamHI	CGTGGATCCTATTTCTCGTGGATGGAGCA	50°C	324 bp
Cia1 DK RP	SpeI	CGTACTAGTCCGGCTATGCTCACCTTCTA	50°C	
Cia2A DK FP	BamHI	CGTGGATCCTCCCAATCCCACTGTCTTTC	58°C	458 bp
Cia2A DK RP	SpeI	CGTACTAGTAGGCATTTACGCATGATTCC	58°C	
Nbp35 DK FP	BamHI	CGTGGATCCAAGGAGGTGTGGGGAAGAGT	58°C	425 bp
Nbp35 DK RP	SpeI	CGTACTAGTAACCATCTGGGGTGTGGTTA	58°C	
Tah18 DK FP	BamHI	CGTGGATCCTGAGAGTGACAGGAAGGGCT	58°C	535 bp
Tah18 DK FP	SpeI	CGTACTAGTCTTCAAAAAGAACCGAAGCG	58°C	

3.1.3 Used plasmid for RNAi construct

For developing the RNAi constructs that were used later to induce RNAi in *Trypanosoma brucei* the transfection plasmid p2T7-177 was used as base vector. The vector contains for the RNAi notable, a 177 bp sequence for targeting to *T. brucei* minichromosomes, two head-to-head T7-promoters, flanking a GFP-fragment inserted in a multiple cloning site, and a phleomycin resistance gene. The vector is designed from an *E. coli* plasmid and in addition to the *E. coli* origin gene sequence it also contains a gene coding for ampicillin resistance to allow a selection of positive transfected *E. coli* cells (Wickstead, Ersfeld & Gull, 2002).

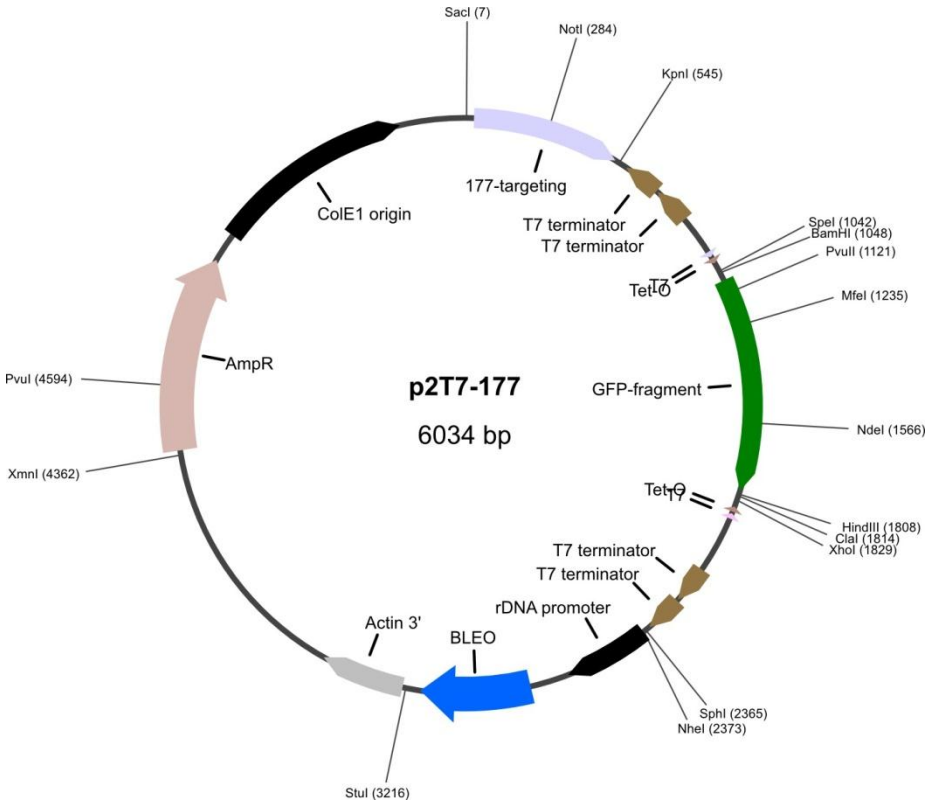


Figure 4: Transfection vector for RNAi: p2T7-177 (Wickstead et al. (2002) MBP 125:211-6)

The following modified version of the p2T7-177 plasmid were used. The GFP-fragment in this plasmid was replaced by fragments of genes of *T. brucei* using the BamHI and XhoI restriction site of the plasmid. They were constructed by Somsuvro Basu, MSc. for investigation of the single RNAi knockdown effects of the genes corresponding to the inserted gene fragments.

p2T7-177-Cia1	GFP-fragment replaced by Cia1-fragment
p2T7-177-Dre2	GFP-fragment replaced by Dre2-fragment
p2T7-177-Nar1	GFP-fragment replaced by Nar1-fragment

3.1.4 Used chemicals and materials

Table 3: List of chemicals used ordered according to the experimental procedure in which they were used

Polymerase Chain Reaction (PCR)	
Genomic DNA of *T. Brucei* extracted at 03.05.2011	
Deoxynucleotide Solution Mix (BioLabs)	10 mM each nucleotide as sodium salt in ultrapure water, pH 7.5
Tag-Purple DNA polymerase (Top-Bio)	1000 U/mL, 20 mM Tris-HCl (pH 8,0 při 25°C), 100 mM KCl, 0,1 mM EDTA, 1

	mM DTT, 0,5% Nonidet P-40, 0,5% Tween 20, inert dye, 50% glycerol
10x PCR Blue buffer complete (Top-Bio)	750 mM Tris-HCl, pH 8,8 (25°C), 200 mM $(NH_4)_2SO_4$, 1% Tween 20, 25 mM $MgCl_2$
2x PPP Master Mix (Top-Bio)	150 mM Tris-HCl, pH 8,8 (25°C), 40 mM $(NH_4)_2SO_4$, 0,02% Tween 20, 5 mM $MgCl_2$, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 400 µM dTTP, 100 U/ml Taq DNA polymerase, dye, stabilizers and additives
5x FIREPol® Master Mix Ready to Load (Solis BioDyne)	FIREPol® DNA polymerase, 5x Reaction Buffer B (0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 0.1% w/v Tween-20*)*, 7.5 mM $MgCl_2$, 2 mM dNTPs of each, Blue dye *(*Migration equivalent to 3.5-4.5 kb DNA fragment*)*, Yellow dye *(*Migration rate in excess of primers in 1% agarose gel: <35-45 bp), Compound that increases sample density for direct loading
Agarose Gel Electrophoresis	
Agarose	
Ethidium Bromide solution	5 mg / mL Ethidium bromid in MiliQ
10x Agarose gel electrophoresis buffer	670 mM Tris-Base, 222mM boric acid, 5 mM EDTA
6X MassRuler™ DNA Loading Dye	10 mM Tris-HCl (pH 7.6) 0.03% bromophenol blue, 60% glycerol, 60 mM EDTA
1 kb DNA Ladder (Invitrogen)	
PCR Clean-Up	
GenElute™ PCR Clean-Up (Sigma-Aldrich)	
Restriction Digestion	
Restriction Enzyme BamHI (BioLabs)	20000 U/mL, 10 mM Tris-HCl, 50 mM KCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 200 µg/ml BSA, 50% Glycerol, pH 7.4 at 25°C
Restriction Enzyme SpeI (BioLabs)	10000 U/mL, 10 mM Tris-HCl, 250 mM NaCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 200 µg/ml BSA, 50% Glycerol, 0.15% Triton X-100, pH 7.4 at 25°C
Restriction Enzyme XhoI (BioLabs)	20000 U/mL, 10 mM Tris-HCl, 50 mM KCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 200 µg/ml BSA, 50% Glycerol, pH 7.4 at 25°C
Restriction Enzyme NotI (BioLabs)	10000 U/mL, 20 mM Tris-HCl, 200 mM NaCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 200 µg/ml BSA, 50% Glycerol, 0.15% Triton X-100, pH 7.4 at 25°C
Restriction Enzyme SphI-HF (BioLabs)	10000 U/ml, 10 mM Tris-HCl, 100 mM NaCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 100 µg/ml BSA, 50% Glycerol,

	pH 7.4 at 25°C
10x NEBuffer 3 (BioLabs)	1000 mM NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM Dithiothreitol, pH 7.9 at 25°C
10x NEBuffer 4 (BioLabs)	500 mM potassium acetate, 200 mM Tris-acetate, 100 mM Magnesium Acetate, 10 mM Dithiothreitol, pH 7.9 at 25°C
100x Bovin Serum Albumin (BioLabs)	10 mg/ml BSA, 20 mM KPO_4, 50 mM NaCl, 0.1 mM EDTA, 5% Glycerol, pH 7.0 at 25°C
Ligation	
T4 DNA Ligase (Invitrogen)	1000 U/ml, 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1mM DTT, 50% (v/v) glycerol
5x T4 DNA Ligase Buffer (Invitrogen)	250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000
Transformation	
Super Optimal broth with Catabolic repression (SOC medium)	2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0 at 25°C
Lysogeny broth (LB) agar plates with Ampicillin	1% trypton, 1% NaCl, 0.5% yeast extract, 1% agar, 100 µg/ml Ampicillin
Ampicillin	25 mg/mL
Lysogeny Broth (LB) medium	1% trypton, 1% NaCl, 0.5% yeast extract
Plasmid DNA isolation	
QIAprep™ Spin Miniprep Kit (Qiagen	
High pure Plasmid Isolation Kit (Roche)	
Gel Extraction Kit	
QIAquick™ Gel Extraction Kit (Qiagen)	
Agarose Gel DNA Extraction Kit (Roche)	
Electroporation of procyclic *T. brucei*	
CytoMix	25 mM HEPES, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 2 mM EDTA, 6 mM Glucose, 5 mM $MgCl_2$, pH 7.6
Electroporation of bloodform *T. brucei*	
AMAXA nucleofector II kit (Lonza)	
Growth of *T. brucei*	
SDM-79 + FBS medium (for procyclics)	Composition according to Brun & Schonenberger, 1979 + 10% fetal bovine serum
HMI – 9 + FBS medium (for bloodform)	Composition according to Hirumi & Hirumi, 1989 + 10% fetal bovine serum
Geneticin (G418)	15 mg/ml in MiliQ
Hygromycin (Hyg)	50 mg/ml in MiliQ
Phleomycin (Phleo)	2.5 mg/ml in MiliQ
Tetracycline	1 mg/ml in MiliQ

3.2 Methods

3.2.1 Generation of Cia2B RNAi single knockdown constructs

The Cia2B single knockdown plasmid was generated by replacing the GFP fragment in the p2T7-177 vector by a fragment of the Cia2B gene. The Cia2B fragment was amplified by PCR. The amplified fragment as well as the p2T7-177 vector where digested with XhoI and BamHI. The digested DNA pieces were ligated together generating the p2T7-177-Cia2B vector. The vector was amplified in *E. Coli* and extracted for use in further applications.

3.2.1.1 Amplification of Cia2B fragment using PCR

The Cia2B fragment was amplified form genomic DNA of T. brucei using the Cia2B SK FP and the Cia2B SK RP (Table 2). Following quantities were used for one PCR reaction.

Table 4: Composition of PCR reaction for amplification of Cia2B fragment

genomic DNA	0.5 µL
Cia2B SK FP	0.5 µL
Cia2B SK RP	0.5 µL
Deoxynucleotide Solution Mix	0.5 µL
10x PCR Blue buffer complete	2.5 µL
Tag-Purple DNA polymerase	0.5 µL
MiliQ	20 µL
Total	25 µL

5 reaction mixtures with 25 µL were prepared. As control of a mixture with the same composition except without genomic DNA was used. Following PCR program was used on a Biometra T3000 Thermocycler:

Table 5: PCR program for amplification of Cia2B fragment

Hot-Start	94°C	3 min 0 sec	
Separation	94°C	0 min 30 sec	
Annealing	58°C	0 min 30 sec	
Elongation	72°C	1 min 0 sec	2↑ 35 cycles
Final Elongation	72°C	8 min 0 sec	
End	16°C	pause	

3.2.1.2 Control of PCR by Agarose Gel Electrophoresis

For controlling the correct amplification of the Cia2B fragment in the PCR reaction, part of the reaction mixture was analyzed by Agarose gel electrophoresis using a 0.75 % Agarose gel containing 0.1 µg/mL EtBr. For preparing of a 0.75 % agarose gel, 0.450 g of agarose were dissolved in 60 ml 1x Agarose gel electrophoresis buffer by heating the solution in the microwave till it nearly boiled and all the Agarose was dissolved. The solution was cooled down to about 55°C under cold water. 1.2 µL of EtBr solution were added to the gel solution and after short mixing it was poured into an OWL Separation System B1 gel cassette with a 1.5 mm 14 well comb inserted. 1 µL of PCR reaction solution was mixed with 1 µL DNA loading dye and loaded on the gel. 5 µL 1Kb DNA ladder were loaded as marker. The gel electrophoresis was performed at 75 V till the DNA migrated into the gel and then the applied voltage was changed to 125 V. Upon completion the DNA bands were visualized under UV-light on a HP Alphaimager.

Table 6: Loading order of agarose gel for check of Cia2B amplification by PCR

Well	1	2	3	4	5	6	7
Sample	Cia2B PCR 1	Cia2B PCR 2	Cia2B PCR 3	Cia2B PCR 4	Cia2B PCR 5	Cia2B PCR Control	1 kb DNA Ladder
Volume	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	5 µL

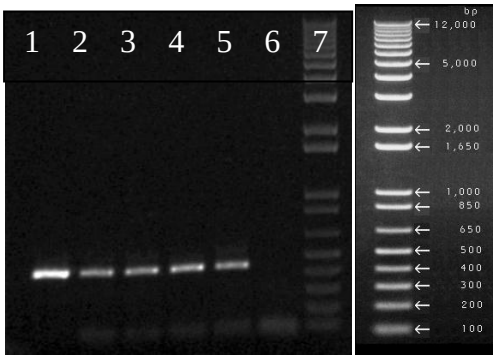


Figure 5: Agarose gel for check of Cia2B amplification (left), and 1 kb DNA ladder (right)

The target size of the amplified gene fragment is 418 bp which corresponds to the observed band in the agarose gel.

3.2.1.3 PCR Clean-up of Cia2B amplification

The five 25 µL PCR solutions were combined, and the DNA was extracted using the GenElute™ PCR Clean-Up (Sigma-Aldrich) following the associated protocol. In short, the

separation column was assembled by placing a GenElute plasmid mini spin column into a provided collection tube. The column was equilibrated for the extraction by adding 0.5 mL of the provided Column Preparation Solution to the mini spin column and spinning it afterwards at 12000 g for 30 sec on an Eppendorf tabletop centrifuge, and discarding the flow through afterwards. The combined PCR solutions were mixed with, 5 volumes of Binding solution to 1 volume of PCR mixture which corresponds to, 625 µL Binding solution, and the mixture was spinned in two steps through the column, using 375 µL in each step and spinning at maximum speed for 1 min, and the flow through was again discarded. The column and the bound DNA was washed by adding 0.5 mL of diluted Wash Solution and spinning it for 1 min at maximum speed. After removing the flow through the column was spinned for another 2 min at maximum speed and an empty collection tube to remove remaining traces to washing solution. Further the clean column was placed into a new 1.5 mL micro-centrifugation tube and 50 µL MiliQ were placed onto the center of the column. After an incubation time of 1 min at room temperature the column was spinned at maximum speed for 1 min. To check if collected flow through contained DNA, 1 µL of solution was used to measure its DNA concentration using NanoDrop (Thermo SCIENTIFIC). The Eppendorf tube containing the DNA was stored at -20°C to conserve it for further use.

3.2.1.4 Restriction of Plasmid and PCR Products

Both cleaned PCR product and p2T7-177 plasmid were digested with BamHI and XhoI in a form of a double digestion. Digestion mixtures were prepared with composition according to following table.

Table 7: Composition of mixtures for restriction digestion of p2t7-177 plasmid and Cia2B fragment

	p2T7-177	Cia2B
DNA	20 µL (~2000 ng)	40 µL (~4000 ng)
XhoI	1 µL	1 µL
BamHI	1 µL	1 µL
NEBuffer 3	5 µL	5 µL
BSA	0.5 µL	0.5 µL
MiliQ	22.5 µL	2.5 µL
Total	50 µL	50 µL

The mixtures were incubated overnight (> 8 h) at 37°C, and was used immediately for further work-up after incubation.

3.2.1.5 Clean-up of Restriction digestion

The digestion product of the p2T7-177 plasmid was separated using agarose gel electrophoresis, and the desired DNA fragment (one of the two fragments formed upon restriction digestion) had to be extracted from the gel. In case of the digestion of the PCR product the pieces cut off from the desired fragment have a length of about 6 to 9 bp, and therefore the DNA can be cleaned using the PCR clean-up kit, because the affinity column provided with the kit doesn't bind DNA fragments smaller than 100 bp.

3.2.1.5.1 Agarose Gel Electrophoresis of p2T7-177 digestion

The 50 µL of the p2T7-177 double digestion were mixed with 10 µL DNA Loading Dye and loaded in a 0.75% agarose gel with a 1.5 mm 10 well comb.

Table 8: Loading of agarose gel with p2T7-177 digestion mixture

Well	1	2	3	4
Sample	Marker	X	p2T7-177	p2T7-177
Volume	5 µL	empty	30 µL	30 µL

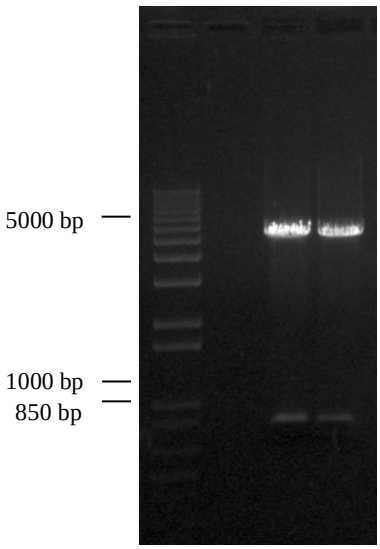


Figure 6: Agarose gel of p2T7-177 digestion

The bands approximately match the fragments generated by the digestion, one with 781 bp resembling the cut out GFP fragment in the p2T7-177 plasmid, the second band is the other piece of the plasmid with a size of 5253 bp.

3.2.1.5.2 Gel extraction of digested p2T7-177 from agarose gel

The desired bands were cut out under UV-light with a sharp scalpel and separated from the rest of the gel. The DNA was extracted using the QIAquick™ Gel Extraction Kit following the associated protocol. In short, the cut gel was weighted and 3 times the amount of Buffer BG was added and the mixture was incubated for 10 min at 50°C with occasional shaking every 2 to 3 min. After complete dissolving of the gel 1 gel volume of isopropanol was added and the DNA was collected by spinning the solution through a QIAquick spin column at maximum speed for 1 min. The flow through was discarded and for washing of the bound DNA 0.75 mL of Buffer PE were added to the column and it was spinned again for 1 min at maximum speed. After removing of the flow through, the column was spinned for an additional minute to remove remaining traces of the Buffer PE. After that the column was placed into a new clean 1.5 mL micro-centrifuge tube and 50 µL MilliQ H_2O were dropped exactly onto the QIAquick membrane. After a 1 min incubation at room temperature the column was spinned for 1 min at maximum speed to collect the eluted DNA. 1 µL of the collected flow through were used to measure the DNA concentration using NanoDrop (Thermo Scientific), and the remaining flow through was stored at -20°C.

3.2.1.5.3 PCR Clean-up Kit for clean-up of Cia2B restriction digestion

The restricted Cia2B fragment was extracted using the GenElute™ PCR Clean-Up kit following the associated protocol (analogue to procedure in 3.2.1.3). For the elution step 50 µL MiliQ were used. 1 µL of the eluate was used to measure the concentration on the Thermo NanoDrop 1000, and the remaining solution was stored at -20°C in the freezer.

3.2.1.6 Ligation and transformation in E. coli

The digested Cia2B fragment was ligated with the p2T7-177 fragment which was extracted from the agarose gel generating the p2T7-177-Cia2B vector. The ligation product was transformed into *E. coli* for amplification.

3.2.1.6.1 Ligation of Cia2B fragment with p2T7-177 fragment

For ligation of the Cia2B and the p2T7-177 fragment that were obtained after the digestion with BamHI and XhoI, following quantities of reagents and DNA were mixed:

Table 9: Composition of mixture for ligation of Cia2B with p2T7-177

100ng p2T7-177 fragment	6.8 µL
30ng Cia2B fragment	0.7 µL
T4 DNA Ligase	0.5 µL
5x T4 DNA Ligase Buffer	4 µL
MiliQ	8 µL
Total	20 µL

The mixture was incubated for 1.5 h at room temperature (20°C). Upon completion of the ligation the solution was diluted to 100 µL with MiliQ. Part of the diluted mixture was immediately used for the transformation in *E. coli*, the remaining solution was stored at -20°C in the freezer.

3.2.1.6.2 Transformation of Ligation product into E. coli

Competitive cells (*E. Coli* XL-1 Blue, stored at -80°C) where thawed for 20 min in ice, then 20 µL of the ligation solution were added to 90 µL of bacteria suspension and the suspension was kept for another 20 min in ice. As control 90 µL bacteria without added DNA was used and treated in the same way as the other bacteria. The bacteria were heat shocked at 42°C for 1 min, and afterwards kept for 2 min on ice. 100 µL of SOC media were added to each vial with bacteria and the vial were shaken for 45 min at 37°C and 200 rpm. 100 µL of the solution was spread onto a LB agar plate with ampicillin. The plates were incubated for 12-16h at 37°C. From each plate 5 colonies were taken and transferred into a test tube with 4 ml of liquid LB media containing 75 µg/mL ampicillin. The tubes were incubated at 37°C for 8 h while shaking at 200 rpm.

3.2.1.7 Conservation of E. coli

From each of the five *E. coli* colonies which were grown in test tubes 800 µL of the liquid media were mixed with 200 µL of 80% glycerol solution in a 1.5 ml Eppendorf tube. These tubes were stored in a -80°C freezer. Some of the liquid media was put into new liquid LB media with ampicillin and grown for 12 h at 37°C and shaking.

3.2.1.8 Extraction of Plasmid DNA

The cells from the liquid media were harvested by centrifuging them on an Eppendorf miniSpin plus tabletop centrifuge. For harvesting the 4 mL liquid media was centrifuged in 750 µL steps in 1.5 mL micro-centrifugation tubes at 10.000 rpm for 30 sec, after each

centrifugation step, the supernatant was discarded and new 750 µL medium from the test tube were added, till in the end all the *E. coli* cells which were grown in the test tube were collected in the centrifugation tube.
The plasmid DNA was isolated using the QIAprep™ Spin Miniprep kit following the associated protocol. In short the harvested bacteria pellet was resuspended in 250 µL Buffer P1, then 250 µL Buffer P2 were added and the solution was mixed by inverting the tube 4 to 6 times or until the solution became clear. After an incubation time of 5 min at room temperature (20°C) 350 µL of Buffer N3 were added and the mixture was homogenized by inverting the tube 4 to 6 times. A white precipitate formed which was collected by centrifuging the tube for 10 min at 13000 rpm. The supernatant was transferred into a QIAprep spin column which was inserted into a 2 mL collection tube. The column was centrifuged for 60 sec at 13000 rpm and the flow-through was discarded. The recommended washing step with the Buffer PB was not performed because *E. coli* XL-1 blue doesn't have a high nuclease activity. Instead the column was immediately washed by adding 0.75 mL Buffer PE and centrifuging for 60 sec at 13000 rpm. After discarding the flow-through the column was centrifuged for an additional minute to remove traces of Buffer PE. After that the column was placed into a new clean 1.5 mL micro-centrifuge tube and 50 µL MIliQ were dropped exactly onto the QIAquick membrane. After an incubation time of 1 min at room temperature the column was spinned for 1 min at maximum speed to collect the eluted DNA. 1 µL of the collected flow through were used to measure the DNA concentration using the Thermo NanoDrop 1000 (Thermo Scientific), and the remaining flow through was stored at -20°C.

3.2.1.9 Check for correctness of the constructed plasmid

First it was checked if the extracted plasmid contains the desired inserted gene fragment. This was done by restriction of the plasmid with the same enzymes which were used to generate the sticky ends for the ligation and by PCR with the same primers used for the amplification of the gene fragment from genomic DNA. Afterwards the plasmids were sent for sequencing to check the correctness of the inserted *T. brucei* gene fragment.

3.2.1.9.1 Check of Plasmid by PCR

For the check by PCR the same primer combination as for the amplification of the Cia2B gene fragment (3.2.1.1.) was used. Five reaction mixtures were prepared, one for each

of the grown *E. coli* colonies which were picked after transfection with the p2T7-177-Cia2B plasmid.

Table 10: Composition of PCR reaction for amplification of Cia2B fragment from p2T7-177-Cia2B plasmid

Plasmid DNA	1 µL
Cia2B SK FP	0.5 µL
Cia2B SK RP	0.5 µL
5X Firepol Master Mix	5 µL
MiliQ	18 µL
Total	25 µL

Because the DNA solution obtained from the plasmid extraction was high concentrated the solution was diluted 1:10 with MiliQ before it was used for the PCR mixture. The PCR was run under the same program as used for the amplification of the Cia2B fragment from genomic DNA (Table 5) but only with 30 cycles instead of 35. For the agarose gel electrophoresis 5 µL of PCR reaction solution were loaded onto a 0.75% agarose gel with containing 0.1 µg/mL EtBr and separated at 125 V.

Table 11: Loading order of agarose gel for check of Cia2B insertion in p2T7-177-Cia2B plasmid

Well	1	2	3	4	5	6	7
Sample	1 kb DNA Ladder	p2T7-177-Cia2B 1	p2T7-177-Cia2B 2	p2T7-177-Cia2B 3	p2T7-177-Cia2B 4	p2T7-177-Cia2B 5	PCR Control
Volume	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL

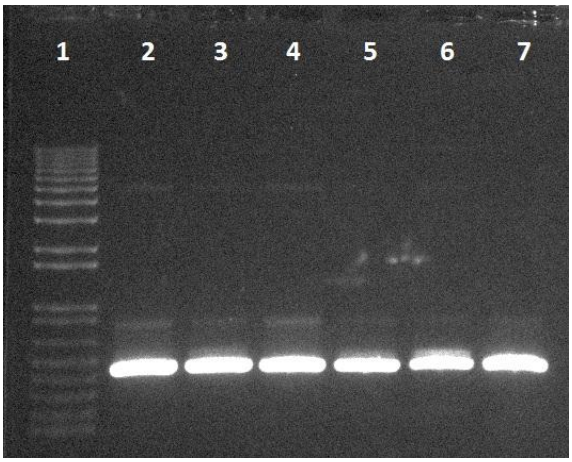


Figure 7: Agarose gel for check of Cia2B insertion in p2T7-177 plasmid

The target size of the amplified gene fragment is 418 bp which corresponds to the observed band in the agarose gel however the control mixture also contained a band which indicated a contamination of one of the used chemicals for the PCR mixture with either

p2T7-177-Cia2B plasmid, traces of the amplified Cia2B fragment or genomic DNA of *T. brucei*, therefore it was decided to additionally carry out a restriction analysis of the p2T7-177-Cia2B plasmid.

3.2.1.9.2 Check of Plasmid by Restriction Digestion

For the check with restriction digestion, a double digestion with the restriction endonucleases BamHI and XhoI was performed. The restriction was only performed for plasmids extracted from the *E. coli* colonies 1 and 3.

Table 12: Composition of mixtures for restriction digestion of p2T7-177-Cia2B plasmid

Plasmid DNA	5 µL
BamHI	0.5 µL
XhoI	0.5 µL
NEBuffer 3	2.5 µL
BSA	0.3 µL
MiliQ	16.7 µL
Total	25 µL

The reaction mixture was incubated at 37°C for 3h. After end of the incubation time 15 µL of the reaction mixture were mixed with 3 µL DNA loading dye, and the digested DNA was fractionated on a 0.75% agarose gel containing 0.1 µg/mL EtBr. The DNA bands were visualized under UV-light on a HP Alphaimager.

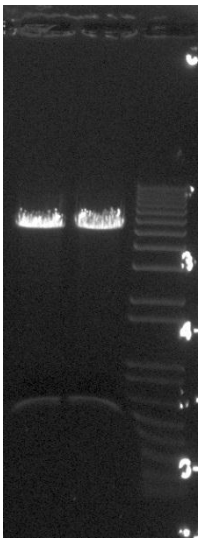


Figure 8: Agarose gel of p2T7-177-Cia2B digestion

The size of the cut out DNA fragment (lower band in figure 8) corresponds to the approximate 418 bp of the inserted gene fragment. This gel confirmed therefore that the plasmid contained the inserted gene fragment and I could send it for sequencing.

3.2.1.9.3 Sequencing of Plasmid

75-150 ng of plasmid DNA were added with 1 µL of Cia2B SK FP and diluted with MiliQ up to 7.5 µL. Similar 75-150 ng of plasmid DNA were added with 1 µL of Cia2B SK RP and diluted with MiliQ up to 7.5 µL. These mixtures were prepared for all 5 plasmids isolated from the 5 different *E. coli* colonies. The sequencing was performed at the institute of Plant Molecular Biology, Biology Centre on an ABI Prism 3130 XL.
The obtained sequence was identified by a BLAST search on NCBI in the nucleotide database of *T. brucei* using nucleotide blast, to check if the gene fragment was present in the plasmid and if it contained any major disparities compared to the original *T. brucei* Cia2B sequence.
The chromatograms obtained from the sequencing were of good quality (data not shown) and confirmed the integrity of the Cia2B fragment which was inserted into the p2T7-177 vector.

3.2.2 Construction of RNAi Double Knockdown plasmids

Double knockdown constructs for RNAi in *T. brucei* had to be constructed containing the gene fragment combinations Cia1-Cia2A, Tah18-Dre2, Cia1-Nar1, Nbp35-Dre2 and Cia2A-Cia2B. For this plasmids of the single knockdowns were transformed and amplified in *E. coli* and afterwards extracted from *E. coli*. Gene fragments designed for RNAi were amplified by PCR from genomic DNA of *T. brucei.* Both single knockdown plasmids and PCR amplified gene fragments were digested sequential with BamHI and SpeI and then ligated together with T4 ligase. The ligated product was transformed into *E. coli* and amplified in it for further experiments.

3.2.2.1 Amplification of gene fragments using PCR

The Cia1, Cia2A, Nbp35 and Tah18 gene fragment for construction of the double knockdown (DK) constructs were amplified by PCR from genomic DNA of *T. brucei* using for each gene the corresponding forward (FP) and reverse primer (RP) (Table 2). Following reaction mixture was prepared 4 times for each primer pair including a control mixture without genomic DNA:

Table 13: Composition of PCR reaction for amplification of *T. brucei* gene fragment

genomic DNA	0.5 µL
Forward primer (FP)	0.5 µL
Reverse primer (RP)	0.5 µL
5X FIREPol Master Mix	5 µL
MiliQ	18.5 µL
Total	25 µL

The PCR was run under the same program as for the amplification of Cia2B (Table 5), the annealing temperature was set different for ach primer according to the temperature noted in table 2. The PCR mixtures were immediately used for further work-up. During the time of the electrophoresis they were stored at 4°C in the fridge.

3.2.2.2 Control of PCR by Agarose Gel Electrophoresis

2 µL of each PCR mixture were run in an agarose gel electrophoresis using a 0.75% agarose gel containing 0.1 µg/mL EtBr. 5 µL 1Kb DNA ladder were loaded as marker. The DNA bands were visualized under UV-light on a HP Alphaimager.
The gels confirmed the correct amplification of the desired gene fragments by matching of the bands with the predicted gene fragment sizes (data not shown).

3.2.2.3 PCR Clean-up of amplified gene fragments

For each gene fragment the 4 PCR mixtures were mixed together and the amplified DNA was extracted using the GenElute^{TM} PCR Clean-Up following the associated protocol (analogue to procedure in 3.2.1.3). For the elution step 50 µL MiliQ were used. 1 µL of the eluate was used to measure the concentration on the Thermo Nanodrop 1000, and the remaining solution was stored at -20°C.

3.2.2.4 Amplification of Vectors in E. coli

To obtain sufficient plasmid DNA for each single knockdown construct to successfully convert into the double knockdown constructs, and compensate for the losses in the involved processing steps, the plasmids had to be amplified in *E. coli*. The single knockdown constructs (mentioned in 3.1.3) were transformed into *E. coli* via heat shock. The bacteria were grown in liquid media to amplify the desired plasmid which later was extracted from the bacteria.

3.2.2.4.1 Transformation of Plasmid

The transformation was performed analogue to the transformation of the ligation product in chapter 3.2.1.6.2, using 20 ng of single knockdown plasmid DNA for the transformation. After an incubation time of the plates for 12-16h at 37°C, from each plate 2 colonies were taken and transferred into a test tube with 4 ml of liquid LB media containing 12 µL ampicillin. The tubes were incubated at 37°C for 8 h while shaking at 200 rpm.

3.2.2.4.2 Isolation of Plasmid DNA

The cells from the liquid media were harvested by centrifuging them at 10.000 rpm for 30 sec in a Eppendorf miniSpin plus tabletop centrifuge.
The plasmid DNA was isolated using the QIAprep™ Spin Miniprep kit following the associated protocol (also see chapter 3.2.1.8). The extracted DNA of both colonies from each single knockdown plasmid were stored at -80°C.

3.2.2.5 Restriction Digestion

Both knockdown plasmids and PCR products were digested first with SpeI and then with BamHI generating 3' and 5' sticky overhangs on both vector and inserts. The restriction products were afterwards purified to serve for a following ligation.

3.2.2.5.1 Restriction of Plasmid and PCR Products with SpeI

Both the cleaned PCR products of the amplification of Cia1, Cia2A, Nbp35 and Tah18 and the RNAi single knockdown plasmids p2T7-177-Cia1; p2T7-177-Dre2; p2T7-177-Nar1 and p2T7-177-Cia2B were restriction digested with SpeI.

Table 14: Composition of mixtures for restriction digestion of p2T7-177 SK plasmids and gene fragment

	insert	plasmid
DNA	20 µL (~2000 ng)	40 µL (~4000 ng)
SpeI	1 µL	1 µL
NEBuffer 4	5 µL	5 µL
BSA	0.5 µL	0.5 µL
MiliQ	23.5 µL	3.5 µL
Total	50 µL	50 µL

The mixtures were incubated overnight (> 8 h) at 37°C, and was used immediately for further work-up after incubation.

3.2.2.5.2 Check of Restriction of plasmids by Agarose Gel Electrophoresis

2 µL of each restriction mixture containing a single knockdown plasmid were mixed with 1 µL DNA loading dye and run in an agarose gel electrophoresis using a 0.75% agarose gel containing 0.1 µg/mL EtBr. 5 µL 1Kb DNA ladder were loaded as marker. The DNA bands were visualized under UV-light on a HP Alphaimager.
The gels confirmed the proper and complete digestion of the plasmids by showing only one band of the linearized plasmid and no remaining band for a circular plasmid (data not shown).

3.2.2.5.3 Clean-up of Restriction Digestion with SpeI

The digestion mixture for each gene fragment and each linearized plasmid was cleaned by extracting the DNA with the GenElute™ PCR Clean-Up kit following the associated protocol (analogue to procedure in 3.2.1.3). For the elution step 50 µL MiliQ were used. 1 µL of the eluate was used to measure the DNA concentration on the Thermo Nanodrop 1000, and the remaining solution was immediately used for the next digestion step.

3.2.2.5.4 Restriction of Plasmid and PCR Products

The 50 µL of DNA solution obtained after the clean-up of the single digestions were each digested with BamHI.

Table 15: Composition of mixtures for restriction digestion single digested DNA with BamHI

DNA	50 µL
BamHI	2.5 µL
NEBuffer 3	12.5 µL
BSA	1.25 µL
MiliQ	58.75 µL
Total	125 µL

The mixtures were incubated overnight (> 8 h) at 37°C, and was used immediately for further work-up after incubation.

3.2.2.5.3 Clean-up of Restriction Digestion with BamHI

The digestion mixture for each gene fragment and each linearized plasmid was cleaned by extracting the DNA the GenElute™ PCR Clean-Up kit, following the associated protocol (analogue to procedure in 3.2.1.3). For the elution step 50 µL MiliQ were used. 1 µL of the eluate were used to measure the DNA concentration on the Thermo Nanodrop 1000, and the

remaining solution containing the double digested DNA fragments were stored for further use at -20°C.

3.2.2.6 Ligation and transformation in E. coli

The double digested gene fragments were ligated with the p2T7-177 single knockdown plasmids in the appropriate combinations to generate the double knock down plasmids p2T7-177-Cia1-Cia2A, p2T7-177-Dre2-Tah18, p2T7-177-Nar1-Cia1, p2T7-177-Cia2B-Cia2A and p2T7-177-Dre2-NBP35. The ligation products were transformed into *E. coli* for amplification of the plasmids.

3.2.2.6.1 Ligation of gene fragments with SK constructs

For ligation of the gene fragments and the p2T7-177 single RNAi knockdown plasmid fragments that were obtained after the digestion with BamHI and SpeI following quantities of reagents and DNA were mixed:

Table 16: Composition of mixture for ligation of gene fragments with p2T7-177 single knock down plasmids

plasmid fragment	100 ng
insert fragment	30 ng
T4 DNA Ligase	0.5 µL
5x T4 DNA Ligase Buffer	4 µL
MiliQ	up to 20 µL
Total	20 µL

The volume of plasmid and insert solution and therefore also the added amount of MiliQ depended on the DNA concentrations of the used plasmid and insert solutions. The mixtures were prepared with the following combinations of insert gene fragment and single knock down plasmids:

Table 17: Ligation combinations of insert gene fragments and single knock down plasmids

Insert	Cia2A	Tah18	Cia1	Cia2A	NBP35
Plasmid	p2T7-177-Cia1	p2T7-177-Dre2	p2T7-177-Nar1	p2T7-177-Cia2B	p2T7-177-Dre2
Resulting vector	p2T7-177-Cia1-Cia2A	p2T7-177-Dre2-Tah18	p2T7-177-Nar1-Cia1	p2T7-177-Cia2B-Cia2A	p2T7-177-Dre2-NBP35

The mixture was incubated for 1.5 h at room temperature (20°C). After finishing of the ligation the solution was diluted to 100 µL with MiliQ. Part of the diluted mixture were immediately used for the transformation in *E. coli*, the remaining solution was stored at -20°C.

3.2.2.6.2 Transformation of Ligation product into E. coli

The transformation was performed analogue to the transformation of the ligation product in chapter 3.2.1.6.2. After an incubation time of the plates for 12-16h at 37°C, from each plate 5 colonies were taken and transferred into a test tube with 4 ml of liquid LB media containing 12 µL ampicillin. The tubes were incubated at 37°C for 8 h while shaking at 200 rpm.

3.2.2.6.3 Conservation of E. coli

From each of the 5 *E. coli* colonies which were grown in test tubes 800 µL of the liquid media were mixed with 200 µL of 80% glycerol solution in a 1.5 ml Eppendorf tube. These tubes were stored in a -80°C freezer. Some of the liquid media was put into new liquid LB media with ampicillin and grown for 12 h at 37°C and shaking.

3.2.2.6.4 Extraction of Plasmid DNA

The cells from the liquid media were harvested by centrifuging them at 10.000 rpm for 30 sec in an Eppendorf miniSpin plus tabletop centrifuge.
The plasmid DNA was isolated using the QIAprep™ Spin Miniprep kit following the associated protocol (also see chapter 3.2.1.8). The extracted DNA of all five colonies from each double RNAi knockdown plasmid was stored at -80°C.

3.2.2.6.5 Check for correctness of the constructed double knock down plasmid

To check if the gene fragments were correct inserted into the single knockdown plasmids, for each plasmid extracted from the picked *E. coli* colonies a PCR check was conducted using the primers of the two inserted gene fragments and a restriction digestion using the restriction enzymes SpeI and SpHI. After confirmation of the integrity of the plasmids they were send for sequencing as final check.

3.2.2.6.5.1 Check of Plasmids by Restriction Digestion

For the check with restriction digestion, a double digestion with the restriction endonucleases SpeI and SpHI-HF was performed.

Table 18: Composition of mixtures for restriction analysis of double knockdown plasmids

Plasmid DNA	150-200 ng
SpeI	0.5 µL
SpHI-HF	0.5 µL
NEBuffer 4	5 µL
BSA	0.5 µL
MiliQ	Up to 25 µL
Total	25 µL

The reaction mixture was incubated at 37°C for 3h. After end of the incubation time 15 µL of the reaction mixture were mixed with 3 µL DNA loading dye, and the digested DNA was fractionated on a 0.75% agarose gel with EtBr. DNA bands were visualized under UV-light on a HP Alphaimager. The sizes of all observed bands matched with the expected sizes of the by the restriction produced plasmid fragments (data not shown).

3.2.2.6.5.2 Check of Plasmid by PCR

Three PCR mixtures were prepared for each plasmid extracted from all five picked *E. coli* colonies for each constructed double knockdown plasmid. One mixture was prepared containing forward and reverse primer of the first insert, and one containing forward and reverse primer of the second insert. A third mixture was prepared containing the forward primer of one insert and the reverse primer of the second insert in such combination that a DNA fragment which contains both gene inserts gets amplified. PCR mixtures were prepared analogue to table 10 and the plasmid DNA was diluted 1:10 with MiliQ before it was used for the PCR mixture. The PCR was run using the same program as in table 5 but only with 30 instead of 35 cycles and the lowest annealing temperature of both primers was used if they were different. Also for each primer combination a blank mixture without plasmid DNA was prepared. After running the PCR 5 µL of each PCR mixture were analyzed by agarose gel electrophoresis using a 0.75% agarose gel containing 0.1 µg/mL EtBr. The DNA bands were visualized under UV-light on a HP Alphaimager. All PCR mixtures prepared with only single and forward primer of one insert resulted in a band of the right size of the amplified fragment. All PCR reactions containing the forward primer of one insert and the forward primer of the second insert resulted in various unspecific amplified DNA bands indicating that either the primer combinations were chosen wrong or the PCR program was unsuitable for the increased length of the intended amplified fragment. (data not shown)

3.2.2.6.5.3 Sequencing of Plasmids

75-150 ng of each RNAi double knockdown plasmids were added with 1 µL of forward primer of the first insert and diluted with MiliQ up to 7.5 µL. Similar 75-150 ng of plasmid DNA were added with 1 µL of the reverse primer of the first insert and diluted with MiliQ up to 7.5 µL. The same mixtures were prepared for each plasmid with the primers of the second insert. These mixtures were prepared for all 5 plasmids isolated from the 5 different *E. coli* colonies for all 5 double-knockdown constructs.
The obtained sequences were identified by a BLAST search on NCBI in the nucleotide database of *T. brucei* using nucleotide blast, to check if the gene fragment was present in the plasmid and if it has contained any major disparity compared to the original *T. brucei* gene sequences.
The chromatograms obtained from the sequencing were of good quality (data not shown) and confirmed the integrity of all gene fragments which were inserted into each of the constructed double knockdown plasmids. The BLAST searches also showed that part of the sequences contained the two targeted genes of *T. brucei* in tandem corresponding to the inserts of the analyzed plasmid, showing the correct build-up of the plasmids, which I was not able to show with the PCR reaction containing forward primer of one insert and reverse primer of the second insert.

3.2.3 RNA interference (RNAi)

To conduct RNAi experiments in *T. brucei* procyclics and blood stream forms the required plasmids first had to be amplified in *E. coli* to obtain sufficient amount of DNA needed for the successful transfection of the *T. brucei* cells. The plasmids were harvested using MIDI-Prep Kit and were linearized by the restriction enzyme NotI. The linearized plasmids were electroporated into the *T. brucei* cells and after 7 to 14 days successful transfected cell lines (identified by antibiotic resistance) were selected. The selected cell lines were grown for 9 to 12 days, one culture induced with tetracycline and another one non-induced and the growth of the cells was measured every day to record a growth curve.

3.2.3.1 Preparation of DNA for RNAi

Approximately 10 µg of plasmid DNA are needed for a successful electroporation of *T. brucei* with reasonable number of positive transfectants. Therefore *E. coli* containing the double knockdown plasmids were thawed from the stock and grown for an MIDI-Prep plasmid extraction. The extracted DNA then was linearized for the electroporation.

3.2.3.1.1 Growing of transfected E. Coli

For each double knockdown the glycerol stocks of one *E. coli* colony containing the desired plasmid, whichs integrity was confirmed by sequencing, was thawn, and 10 µL of the liquid were transferred into 4 mL liquid LB media with 12 µL ampicillin. The tubes were incubated at 37°C for 8 h while shaking at 200 rpm. 1 mL of these starter cultures were transferred to new 25 mL of liquid LB media with added 75 µL ampicillin solution. The cells were grown for 12 h at 37°C while shaking at 200 rpm.

3.2.3.1.2 Harvesting of Plasmid DNA by MIDI-Prep

The *E. coli* cells grown in the 25 mL liquid media were harvested by centrifuging them at 6000 g for 15 min at 4°C. The plasmid DNA in the cells was extracted using a QIAGEN MIDIprep kit according to the provided procedure. In short, the bacteria pellets were resuspended in 4 mL of Buffer P1, then 4 mL Buffer P2 were added and the solution was mixed by inverting for 4 to 6 times. After an incubation time of 5 min at room temperature 4 mL of chilled Buffer P3 were added and the solution was mixed again by inverting 4 to 6 times. After incubating for 15 min on ice the solution was centrifuged at 20000 g for 30 min at 4°C. The supernatant was transferred into a new centrifugation tube and centrifuged for another 15 min at 20000 g and 4°C. In the meanwhile a QIAGEN-tip 100 was equilibrated by allowing 4 mL of Buffer QBT to flow through by gravity flow. Afterwards the supernatant from the centrifugation was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed 2 times with 10 mL Buffer QC. The DNA was eluted with 5 mL Buffer QF, and 3.5 mL of isopropanol were added to the eluted DNA to precipitate it. The DNA was collected in a pellet by centrifugation at 15000 g for 30 min at 4°C. The supernatant was discarded and the pellet was washed with 2 mL 70% ethanol. After another centrifugation step for 10 min at 15000g at room temperature the supernatant was discarded and the pellet was air dried for 10 min. The dry DNA pellet was re-dissolved in 500 µL MiliQ and the concentration of the resulting DNA solution was determined using Nanodrop. The DNA solution was stored at -20°C

3.2.3.1.3 Linearization of Plasmids

To linearize the double knockdown plasmids for the electroporation 12000 ng of each plasmid were linearized using the restriction enzyme NotI. For each double knockdown plasmid two digestion mixtures were prepared, one for the electroporation of bloodstream and one for the electroporation of procyclic *T. brucei*. A digstion mixture contained following composition:

Table 19: Composition of digestion mixtures for linearization of double knockdown plasmids for electroporation

Plasmid DNA	12000 ng
NotI	3 µL
NEBuffer 3	15 µL
BSA	1.5 µL
MiliQ	Up to 150 µL
Total	150 µL

The mixture was incubated at 37°C overnight. On the next day the successful linearization of the plasmids was confirmed by mixing 2 µL of digestion mixture with 1 µL DNA loading dye and performing an agarose gel electrophoresis using a 0.75% agarose gel containing 0.1 µg/mL EtBr. The linearized plasmid of the remaining digestion mixture was extracted using a PCR Clean-up kit (analogue to 3.2.1.3) so that 50 µL DNA solution were obtained. The DNA concentration of the eluate obtained from the PCR clean-up kit was measured on NanoDrop to check if still sufficient DNA (approximately 10 µg) was available for the electroporation.

3.2.3.2 Electroporation

For the transformation of the linearized double knockdown plasmids, wild type *T. brucei* 29.13 of procyclic and bloodstream stage were electroporated with the plasmid DNA according to the appropriate protocols, and after certain time of recovering from the stress of electroporation, positive transfectants were selected using the antibiotics resistances encoded in the double RNAi knockdown plasmids.

3.2.3.2.1 Electroporation of Trypanosoma procyclic stage

For the electroporation of procyclic *T. brucei*, cells were grown in 50 mL SDM-79 medium (see table 3), containing 15 µg/mL Geneticin (G418) and 50 µg/mL Hygromycin (Hyg), to a concentration of 10 to 20 x 10^6 cells per mL. For one electroporation 10 mL of this solution were spinned for 10 min at 1300 g at 4°C. The resulting cell pellet was washed

once with 10 mL ice cold CytoMix (see table 3) and the cells were spinned down for 10 min at 700 g at 4°C, and afterwards the supernatant was discarded. The cell pellet was resuspended in 400 µL CytoMix and the 50 µL DNA solution obtained from the clean-up of the linearization were added. The solution was mixed by pipetting up and down and was loaded in a ice cold electroporation cuvette with a 0.2 cm gap. The cuvette was inserted into the ECM650 electroporator (BTX) and one pulse with the BTX settings: 1600 V, 25 Ω and 50 µF was applied. The content of the cuvette was pipetted into 5 mL new SDM-79 medium containing 15 µg/mL G418 and 50 µg/mL Hyg. The solution was incubated for 18 h under shaking. After this incubation time the medium was mixed with new SDM-79 medium containing 15 µg/mL G418, 50 µg/mL Hyg and 5 mg/mL Phleomycine (Phleo). The 10 mL solution were pipetted into the first row of a 24 well plate, 1.5 mL per well. In the second, third and fourth row 1 mL, 1 mL and 0.5 mL, respectively, new SDM-79 medium containing 15 µg/mL G418, 50 µg/mL Hyg and 2.5 mg/mL Phleo, were filled. Each well of the first row was used to make an dilution series by pipetting 0.5 mL of the well of the first row across the second and third and finally the fourth well, to prepare cell dilutions of 1/3, 1/9 and 1/18 of the first well. The cells were grown in a 27°C incubator and checked every 24 h for their viability.

3.2.3.2.2 Electroporation of Trypanosoma blood-stream stage

The electroporation for blood-stream stage *T. brucei* was carried out using the AMAXA nucleofector II kit (Lonza). For electroporation of bloodstream form *T. brucei* cells were grown in 150 mL HMI-9 medium (see table 3), containing 2.5 µg/mL Geneticin (G418), to a concentration of 10 x 10^6 cells per mL. 30 million cells are needed for one successful electroporation, therefore for one electroporation 30 mL of the cell culture were spinned down at 1500 rpm for 10 min at room temperature (20°C). The supernatant was discarded and the cell pellet was resuspended in 100 µL AMAXA Human T-cell solution which was kept cool at 4°C. The resuspended cells were mixed with the 50 µL DNA solution obtained from the clean-up of the linearization, and the mixture was pipetted into a electroporation cuvette provided with the kit. The cuvette was placed into the AMAXA nucleofector II and the electroporation was performed using the program X-001. Three falcon tubes containing fresh HMI-9 medium were prepared, tube A containing 30 mL and tube B and C 27 mL medium each. The content of the electroporation tube was transferred to the medium in tube A, and after mixing 3 mL were pipetted into tube B. Tube B was also mixed and 3 mL were pipetted into tube C, therefore generating dilution of 1/10 and 1/100 of

the cell concentration in tube A. The media were pipetted into 24 well plates, one plate per tube, 1 mL medium per well. The plates were incubated at 37°C for 10 h. After this incubation time to each well 1 mL HMI-9 medium containing 5 μg/mL G418 and 5 μg/mL Phleo were added. The cells were further grown at 37°C under 5% CO_2 atmosphere and checked every 24 h for their viability.

3.2.3.2.3 Selection of positive transfectans

For blood-steam cells around 7 days and for procyclic cells after around 14 days after electroporation some wells in the plates were teeming with transfectants that showed resistance to the phleomycin and therefore were positive transfected. For each double knockdown construct 3 cell lines were picked (3 for procyclic and 3 for bloodstream) and transferred to new 24 well plates, 1 plate per cell line. The cells in the plates were continued grown and checked every day. If cell density became too high in the wells, the cell concentration was counted using Z2 cell counter (Beckman Coulter Inc.). Blood-stream cell lines were diluted to $1x10^5$ cells per mL using fresh HMI-9 medium containing 2.5 μg/mL G418 and 2.5 μg/mL Phleo, and procyclics to $1x10^6$ cells per mL using fresh SDM-79 medium containing 15 μg/mL G418, 50 μg/mL Hyg and 2.5 mg/mL Phleo.

3.2.3.3.4 Conservation of transfected Trypanosma

After growing the selected transfectants for 3 days in mid-logphase (cell concentration between $1x10^5$ and $5x10^6$ cells per mL for bloodstream, and between $1x10^6$ and $5x10^7$ cells per mL for procyclic) cells were transferred to 5 mL new medium and grown to a concentration of $1x10^6$ and $1x10^7$ cells per mL respectively for bloodstream and procyclic cell lines. 800 μL of these cell solutions were mixed with 200 μL sterile 80% glycerol in a 1.2 mL cryogenic vial. The vials were placed into a cryo-container filled with glycerol, and the cells were pre-cooled for couple of days in a -80°C freezer. After that period the vials were stored in liquid nitrogen for later use.

3.2.3.4 Growing of Trypanosoma

3.2.3.4.1 Growing of procyclic wild type cells

Wild-type procyclic *T. brucei* 29-13 were grown in SDM-79 medium added with 10% fetal bovine serum and 15 μg/mL Geneticin and 50 μg/mL Hygromycin. The remaining composition of the SDM-79 medium was prepared according to Brun & Schonenberger

(1979). Cells were grown in mid-log phase in a cell density range between $1x10^6$ and $5x10^7$ cells per mL, and diluted every second day to a concentration of $1x10^6$ cells per mL using fresh media. Cell cultivation was performed in an incubator at 27°C.

3.2.3.4.2 Growing of blood-form wild type cells

Wild-type blood-form *T. brucei* 29-13 were grown in HMI-9 medium added with 10% fetal bovine serum and 2.5 μg/mL Geneticin. The remaining composition of the HMI-9 medium was unchanged and according to the recommendations of Hirumi & Hirumi (1989). Cells were grown in mid-log phase in a cell density range between $1x10^5$ and $5x10^6$ cells per mL, and diluted every day to a concentration of $1x10^5$ cells per mL using fresh media. Cell cultivation was performed in an incubator at 37°C and a 5% CO_2 atmosphere.

3.2.3.4.3 Growth curves of RNAi induced procyclic cells

For monitoring of the growth curves of the procyclic double knockdown transfectants, cells were grown in SDM-79 medium containing 15 μg/mL G418, 50 μg/mL Hyg and 2.5 mg/mL Phleo. On the starting day of the growth curve (day 0) two wells of a 24 well plate were filled with 1 mL cell culture which was diluted with a starting concentration of $1x10^6$ cells per mL and one well was induced by 1 μg/mL of tetracycline concentration to. Cell densities were measured every 24 h using a Z2 cell counter (Beckman Coulter Inc.), and cells were diluted to $1x10^6$ cells per mL in a new well every second day. Every time the medium was renewed during dilution the induced cell line was induced freshly by appropriate amount of 1 μL of 1 mg/mL tetracycline solution. Growth curves were recorded for 12 days.

3.2.3.4.4 Growth curves of RNAi induced bloodstream cells

For monitoring of the growth curves of the bloodstream double knockdown transfectants, cells were grown in HMI-9 medium containing 2.5 μg/mL G418, and 2.5 mg/mL Phleo. On the starting day of the growth curve (day 0) two wells of a 24 well plate were filled with 1 mL cell culture which was diluted with to a starting concentration of $1x10^5$ cells per mL and one well was induced by increasing the tetracycline concentration to 1 μg/mL. Cell densities were measured every 24 h using a Z2 cell counter (Beckman Coulter Inc.), and cells were diluted to $1x10^5$ cells per mL in a new well every day. Every time the medium was renewed during dilution the induced cell line was induced freshly by

appropriate amount of 1 mg/mL tetracycline solution. Growth curves were recorded for 9 days.

4 Results and discussion

All double knockdown plasmids were successfully constructed, which was confirmed by sequence analysis. The plasmids were amplified in *E. coli* and upon linearization electroporated into *T. brucei* bloodstream and procyclic form. Positive cell lines were selected by antibiotic Phleomycine. Cells were grown to mid-log phase, and part of them was conserved as glycerol stocks and stored in liquid nitrogen. RNAi experiments were recorded by measuring the growth of cells, where the non-induced cells were compared to RNAi-induced cells. RNAi was initiated by the addition of tetracycline to the growth medium.

4.1 Results obtained from growth curves

Non-induced and tetracycline induced cell lines were grown in parallel for 9 to 12 days and cell density was counted every day. Cells were diluted every day for blood-stream form and every second day for procyclics. To obtain growth curves, the every day growth was summed up to obtain a relative diagram showing the theoretical cell density if cells would not have been diluted.

4.1.1 Growth curves of double knockdowns in procyclics

Following graphs show the growth curves which were recorded for the double knockdowns in procylic *T. brucei*. Non-induced cells and tetracycline induced cells were grown parallel in for 12 days. Cells were counted every day, and were diluted every second day down to a concentration of $1x10^6$ cells per mL. Because the cells grow in a log phase the growth curve was displayed in the decadic logarithm of the cell density.

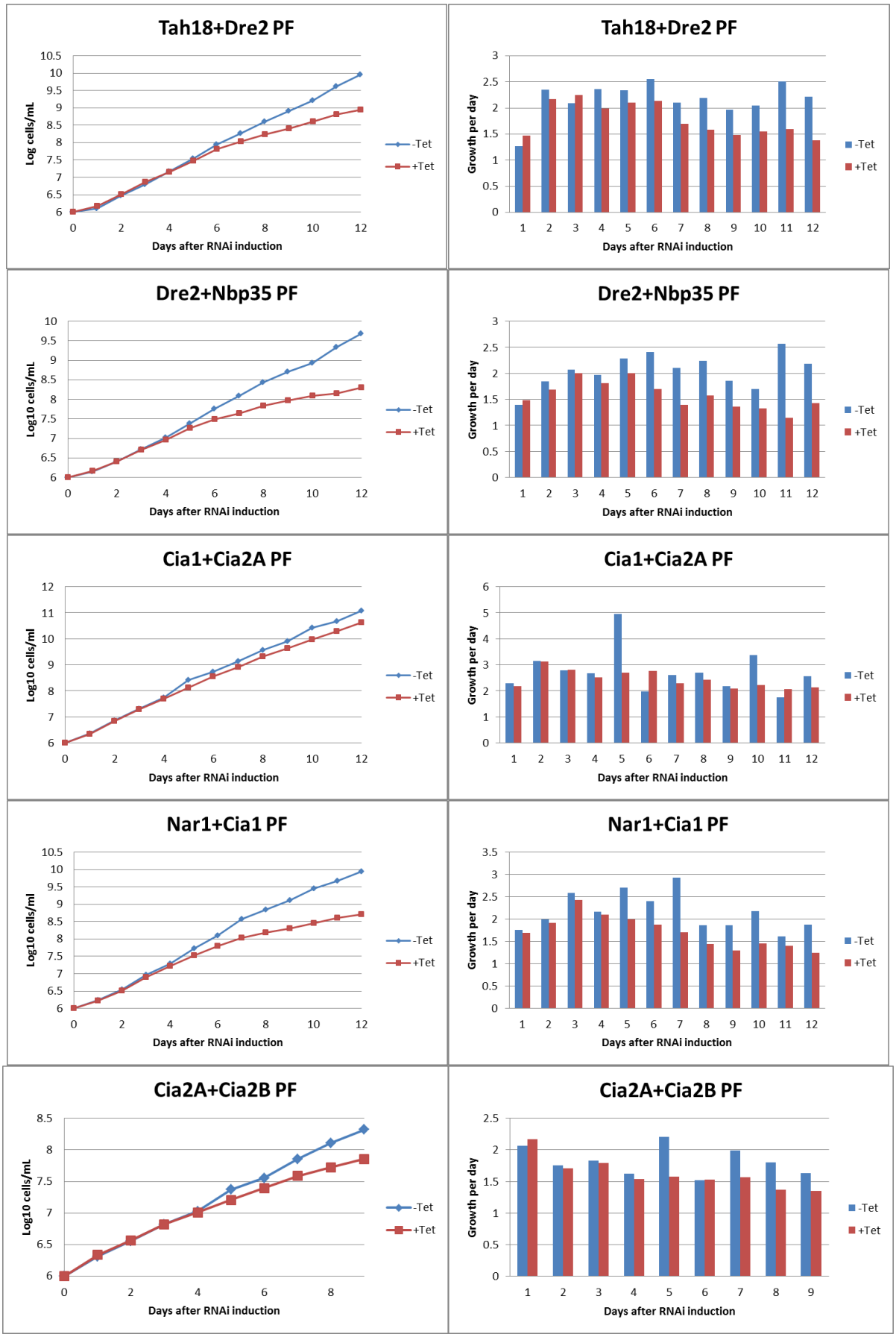


Figure 9: Growth curves recorded for double knockdowns in procyclic *T. brucei* (left) and corresponding measured growth per day (right)

The double knockdown RNAi experiment was aimed to show if the two genes represent essential partners in the CIA pathway. All double knockdown combinations except Cia1+Cia2A showed growth phenotype starting from the day fifth after RNAi induction. The growth curves were compared to the growth curves of the relevant single knockdowns, that were already measured earlier by my co-supervisor Somsuvro Basu, MSc. . The results of the single knockdowns are summarized in table 20 and compared to the results of the double knock-downs.

Table 20: Results of single knockdowns compared to double knockdowns in procyclic *T. brucei*, the diagonal shows the single knockdown experiments, x standing for an observed growth phenotype in the induced cell line, o standing for no growth phenotype

PF	Cia1	Cia2A	Cia2B	Dre2	Nar1	Nbp35	Tah18
Cia1	O	O			X		
Cia2A		O	X				
Cia2B			O				
Dre2				O		X	X
Nar1					O		
NBP35						X	
Tah18							O

The single knockdowns of Cia2A and Cia2B did not show any growth phenotype, the double knock-down however did, and same was true for the combinations Dre2 with Tah18, and Cia1 with Nar1.

Dre2 with Nbp35 also showed a growth phenotype, but it was of similar intensity as observed in the single knock-down of Nbp35. Therefore there was no additional effect on growth by knocking-down those genes in parallel. The additional effect on growth was expected, because Dre2 transports the sulfur equivalent for the [Fe-S] assembly to the Cfd1-Nbp35 complex. Our results indicates that either Dre2 is required in very small amount and still transports the sulfur equivalent to Cfd1, or that Dre2 is completely non-essential and the sulfur for the cluster assembly can also be obtained from other sources of the cytosol.

Cia2B is a homologue of Cia2A and are expected to fulfill similar tasks in the CIA pathway. No growth phenotype was observed by single knockdowns of both genes, which can be explained by their functional redundancy. However in the double knock-down a growth arrest was observed, most likely because the homologues could not replace each other anymore. It is in line with the findings from yeast, where Cia2 is essential for CIA. The possible replacing of Cia2A by Cia2B and *vice versa*, also explains why no growth effect could be seen in the double knock-down of Cia1 and Cia2A.

The double knock-down of Cia1 with Nar1 was of our interest, because earlier studies in yeast indicated that these two proteins form a complex needed for the maturation of the [Fe-S] proteins by the CIA system (Balk, Aquilar Netz, Tepper, Piereik & Lill, 2005). Cia1 was proposed to be the central scaffold of the complex, surrounded by Nar1, Met18, Cia2 in yeast (Weerapana et al, 2010). It was however uncertain if Cia1 or Nar1 is the scaffold, since both proteins have nearly the same size, and none of them showed a growth phenotype in RNAi single-knock-down. Additionally more recent study in human HeLa cells showed that Met18 seems to form the central scaffold of the complex and Nar1 acts as transporter for [Fe-S] between Cfd1-Nbp35 complex and Met18 (Stehling et al, 2012). The double knock-down however showed a growth arrest indicating that both proteins interact in the maturation of the apo
[Fe-S] proteins, and Nar1 transports the cluster to Met18. Cia1 might act as mediator for Nar1 and Met18 binding.

The growth phenotype observed by the double knock-down of Dre2 with Tah18, confirms that they interact and Tah18 can transfer electrons to Dre2 as proposed by Netz et al. (2010).

4.1.2 Growth curves of double knockdowns in blood stream form

Following graphs show the growth curves which were recorded for the double knockdowns in *T. brucei* bloodstream form. Non-induced cells and tetracycline induced cells were grown in parallel for 8-10 days. Cells were counted and diluted, to a concentration of $1x10^5$ cells per mL, every day. Graphs were calculated similarly as in procyclics knock-down experiments.

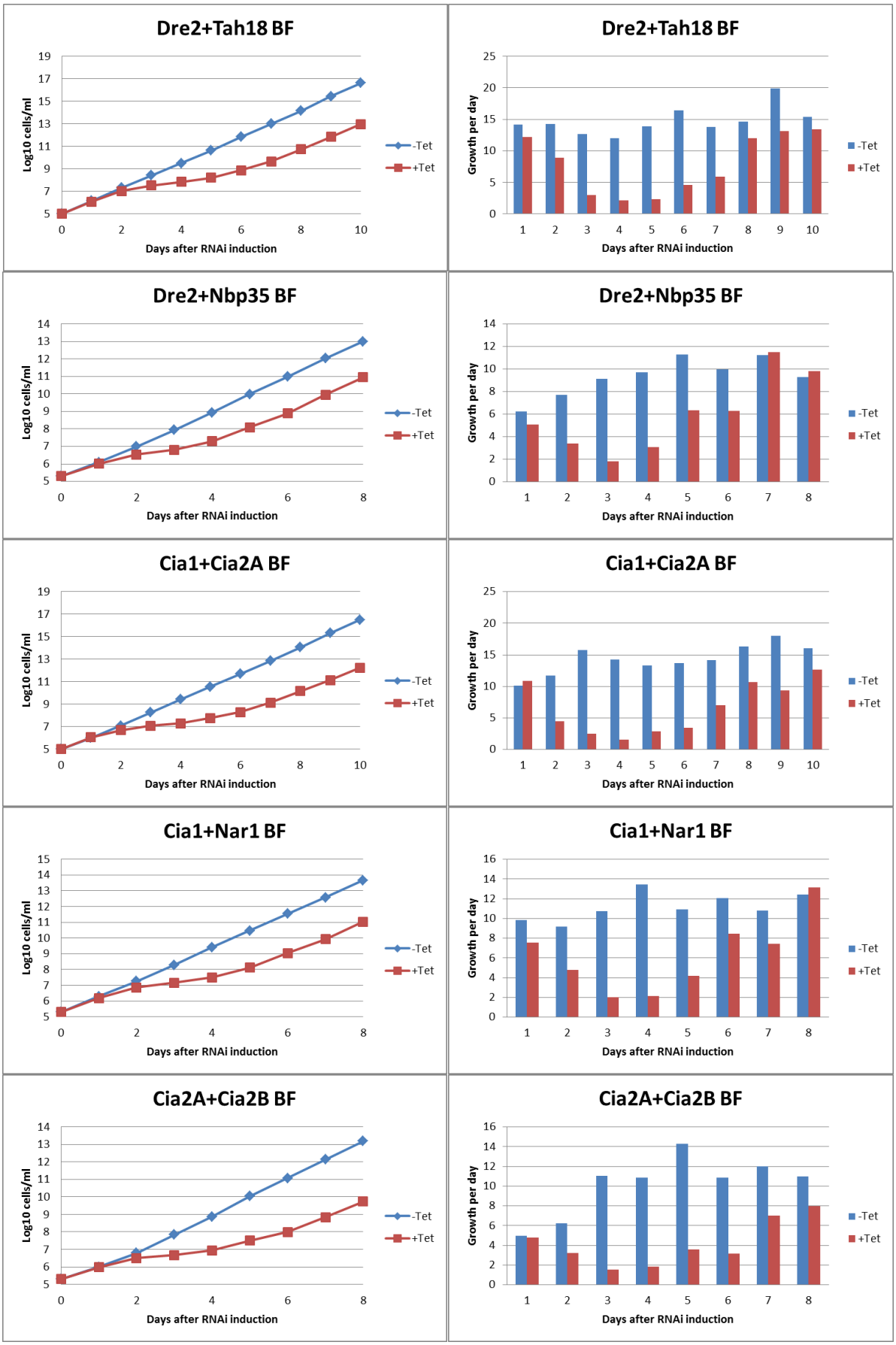


Figure 10: Growth curves recorded for double knockdowns in *T. brucei* blood-stream form (left) and corresponding measured growth per day (right)

Blood-stream form *T. brucei* have in their natural environment a different metabolism than procyclic *T. brucei*. Procyclics, which live in insect fluids, obtain their energy mainly by metabolizing L-proline. In contrast, blood-stream *T. brucei* obtain their energy by glycosylation of glucose, which is available in the blood of the vertebrate host. In the laboratory conditions both forms are usually grown in glucose rich medium and both of them obtain their energy from glycolysis. Blood-stream trypomastigotes however still have a faster metabolism than procyclics, which can be easily seen by comparing the growth of both forms per day. RNAi is therefore observed faster and with more severe phenotypes. The investigation of the blood-stream stage also gives better insights for possible treatments of diseases caused by *Trypanosomes* in human and live-stock.

Table 21: Results of single knockdowns compared to double knockdowns in blood stream form *T. brucei*, the diagonal shows the single knock-down experiments, x standing for an observed growth phenotype in the induced cell line**,** o standing for no growth phenotype

BF	Cia1	Cia2A	Cia2B	Dre2	Nar1	Nbp35	Tah18
Cia1	O	X			X		
Cia2A		X	X				
Cia2B			X				
Dre2				O		X	X
Nar1					O		
NBP35						X	
Tah18							O

All measured RNAi double knock downs showed growth phenotype in blood stream *T. brucei*. For the knock-down combinations Dre2+Tah18, Cia1+Nar1 and Dre2+Nbp35 similar conclusion can be made as in procyclics (see 4.1.4). The growth phenotypes for Cia2B+Cia2A and Cia1+Cia2A are less unsuspected than in procyclics, because already the single knock downs of Cia2A and Cia2B showed growth arrests in blood streams. The double knock down of Cia1 with Cia2A however still showed an increased respond as growth arrest on the second day was already stronger than for Cia2A alone. For the double knock-down of Cia2A with Cia2B also a stronger onset of growth arrest was observed on the second day after induction compared to each single knock-down, however this might only be the additive effects of the two single knockdowns and no additional effect which. More detailed conclusions in blood-streams cannot be made, because of the ability of blood-stream *T. brucei* to recover from RNAi inductions after usually the fourth day of induction, and there is no stable growth arrest as observed in procyclics.

5 Conclusions

Important players in CIA machinery in *S. cerevisiae*, Tah18, Dre2, Nar1, Cia1, Cia2A and Cia2B showed a surprising lack of essentiality in *T. brucei* (except for Cia2A and Cia2B in blood-stream *T. brucei*). Therefore double RNAi knockdowns were designed based on the current working model of the CIA machinery in *S. cerevisia*e, including at least one non-essential gene from *T. brucei* and a second which is predicted to be an interacting partner.

The growth phenotype of the Dre2+Tah18 double knock-down is consistent with the finding from *S. cerevisiae* where these two proteins are interacting partners. Further Cia1 and Nar1 showed, as well as Cia1 and Cia2A showed to be interacting partners. Because the double knock-down of Cia2A and Cia2B in procyclics indicated that these proteins might be also interacting partners or homologues with similar function, we can conclude that Cia1 forms a complex together with Nar1, Cia2A and Cia2B. In humans Met18 was predicted to be scaffold for this complex, unfortunately this gene was not included in our survey. Nevertheless Met18 single knock-down turned out to be not essential in both life cycle stages. Double knockdowns of Met18 with Nar1, Cia1 and Cia2A (or Cia2B) could give further insight into the composition of this complex. All double knockdowns also still have to be investigated for their influence on cytosolic aconitase activity which is a product of the CIA pathway. These results combined should yield additional views on the CIA pathway. However new CIA model proposed based on the results of a Met18 pull down (Stehling et al, 2012), suggests that there are two different pathways of the CIA. According to this model the Met18-complex is not involved in the maturation of c-aconitase, therefore another suitable activity assay would have to be found. A further project in this topic would be to conduct knock out experiments on the CIA components, this would give definite results over the essentiality of the single components in *T. brucei* and also would answer the question if Cia2A and Cia2B are really equivalent homologues and can replace each other.

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